AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology

Allosteric Activation of *Escherichia coli* Glucosamine-6-Phosphate Deaminase (NagB) *In Vivo* Justified by Intracellular Amino Sugar Metabolite Concentrations

Laura I. Álvarez-Añorve,[a,b] Isabelle Gaugué,[a*] Hannes Link,[c*] Jorge Marcos-Viquez,[b] Dana M. Díaz-Jiménez,[b] Sergio Zonszein,[b] Ismael Bustos-Jaimes,[b] Isabelle Schmitz-Afonso,[d*] Mario L. Calcagno,[b] Jacqueline Plumbridge[a]

CNRS-UMR8261, Université Paris Diderot, Sorbonne Paris Cité, Institut de Biologie Physico-Chimique, Paris, France[a]; Laboratorio de Fisicoquímica e Ingeniería de Proteínas, Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico, CDMX, Mexico[b]; Institute of Molecular Systems Biology, ETH Zurich, Zurich, Switzerland[c]; Institut de Chimie des Substances Naturelles, ICSN-CNRS UPR2301, LabEx CEBA, Université Paris-Sud, Gif-sur-Yvette, France[d]

ABSTRACT

We have investigated the impact of growth on glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) on cellular metabolism by quantifying glycolytic metabolites in *Escherichia coli*. Growth on GlcNAc increased intracellular pools of both GlcNAc6P and GlcN6P 10- to 20-fold compared to growth on glucose. Growth on GlcN produced a 100-fold increase in GlcN6P but only a slight increase in GlcNAc6P. Changes to the amounts of downstream glycolytic intermediates were minor compared to growth on glucose. The enzyme glucosamine-6P deaminase (NagB) is required for growth on both GlcN and GlcNAc. It is an allosteric enzyme in *E. coli*, displaying sigmoid kinetics with respect to its substrate, GlcN6P, and is allosterically activated by GlcNAc6P. The high concentration of GlcN6P, accompanied by the small increase in GlcNAc6P, drives *E. coli* NagB ($NagB_{Ec}$) into its high activity state, as observed during growth on GlcN (L. I. Álvarez-Añorve, I. Bustos-Jaimes, M. L. Calcagno, and J. Plumbridge, J Bacteriol 191:6401–6407, 2009, http://dx.doi.org/10.1128/JB.00633-09). The slight increase in GlcNAc6P during growth on GlcN is insufficient to displace NagC, the GlcNAc6P-responsive repressor of the *nag* genes, from its binding sites, so there is only a small increase in *nagB* expression. We replaced the gene for the allosteric $NagB_{Ec}$ enzyme with that of the nonallosteric, *B. subtilis* homologue, $NagB_{Bs}$. We detected no effects on growth rates or competitive fitness on glucose or the amino sugars, nor did we detect any effect on the concentrations of central metabolites, thus demonstrating the robustness of amino sugar metabolism and leaving open the question of the role of allostery in the regulation of NagB.

IMPORTANCE

Chitin, the polymer of *N*-acetylglucosamine, is an abundant biomaterial, and both glucosamine and *N*-acetylglucosamine are valuable nutrients for bacteria. The amino sugars are components of numerous essential macromolecules, including bacterial peptidoglycan and mammalian glycosaminoglycans. Controlling the biosynthetic and degradative pathways of amino sugar metabolism is important in all organisms to avoid loss of nitrogen and energy via a futile cycle of synthesis and breakdown. The enzyme glucosamine-6P deaminase (NagB) is central to this control, and *N*-acetylglucosamine-6P is the key signaling molecule regulating amino sugar utilization in *Escherichia coli*. Here, we investigate how the metabolic status of the bacteria impacts on the activity of $NagB_{Ec}$ and the *N*-acetylglucosamine-6P-sensitive transcriptional repressor, NagC.

The genes for use of *N*-acetylglucosamine (GlcNAc) as a carbon source in *Escherichia coli* form a typical inducible regulon, controlled by the NagC transcription factor. The divergent operon *nagE-nagBACD* (Fig. 1) encodes the transporter (NagE) and the enzymes *N*-acetylglucosamine-6P (GlcNAc6P) deacetylase (NagA) and glucosamine-6P (GlcN6P) deaminase (NagB) for the conversion of GlcNAc6P to fructose-6P (F6P). NagD encodes a UMP phosphatase (UmpH) involved in pyrimidine homeostasis (1). The *E. coli* GlcN6P deaminase enzyme NagB (EC 3.5.99.6) has been extensively studied as a model allosteric enzyme. It is a homohexameric protein that displays positive cooperativity toward its substrate, GlcN6P, and is allosterically activated by GlcNAc6P (2, 3). GlcNAc6P is the substrate of the previous enzyme in the pathway of amino sugar assimilation, GlcNAc6P deacetylase (NagA) (Fig. 1B). Allosteric activation by the substrate of the previous reaction constitutes part of a feed-forward loop. In the case of glycolysis, the function of a feed-forward loop is to sense flux through the pathway (4), and feed-forward regulation in general speeds up the response time to extrinsic signals (5). Several of the enzymes of glycolysis and gluconeogenesis have been shown to

Received 2 November 2015 **Accepted** 15 March 2016

Accepted manuscript posted online 21 March 2016

Citation Álvarez-Añorve LI, Gaugué I, Link H, Marcos-Viquez J, Díaz-Jiménez DM, Zonszein S, Bustos-Jaimes I, Schmitz-Afonso I, Calcagno ML, Plumbridge J. 2016. Allosteric activation of *Escherichia coli* glucosamine-6-phosphate deaminase (NagB) *in vivo* justified by intracellular amino sugar metabolite concentrations. J Bacteriol 198:1610–1620. doi:10.1128/JB.00870-15.

Editor: V. J. DiRita

Address correspondence to Jacqueline Plumbridge, jackie.plumbridge@ibpc.fr.

* Present address: Isabelle Gaugué, CNRS UMR 3215/INSERM U-934, Institut Curie, Centre de Recherche, Paris, France; Hannes Link, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany; Isabelle Schmitz-Afonso, Normandie Université, COBRA, CNRS-UMR6014/FR 3038, Université de Rouen, IRCOF, Mont St. Aignan, France.

L.I.A.-A., I.G., H.L., and J.M.-V. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00870-15.

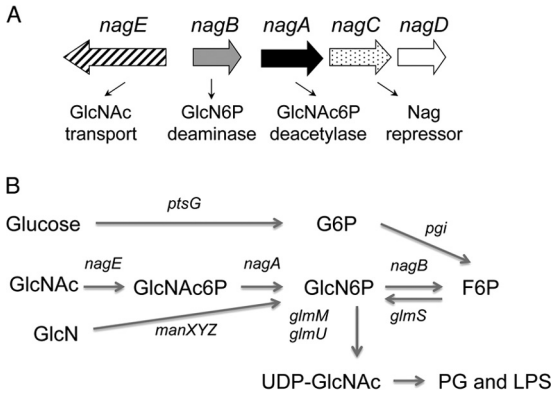


FIG 1 Use of amino sugars by *E. coli*. (A) Organization of the divergent *nagE* and *nagBACD* operons encoding the PTS transporter of GlcNAc (NagE), the enzymes for the metabolism of GlcNAc, GlcNAc6P deacetylase (NagA), and GlcN6P deaminase (NagB), the repressor of the operon (NagC) and NagD, a UMP phosphatase (1). (B) Metabolism of amino sugars. GlcNAc predominantly enters the bacteria by the phosphotransferase system (PTS) transporter, NagE, producing intracellular GlcNAc6P, which is metabolized by the NagA (GlcNAc6P deacetylase) and NagB (GlcN6P deaminase) enzymes to fructose-6P (F6P). GlcN is transported by the *manXYZ*-encoded PTS transporter, producing GlcN6P, which is also metabolized by NagB. Glucose is predominately transported by the *ptsG*-encoded PTS transporter giving G6P and is converted to F6P, the first common compound of the glycolytic pathway. GlcN6P is synthesized by the *glmS*-encoded enzyme and converted, by the sequential action of the *glmM* and *glmU*-encoded enzymes, to UDP-GlcNAc, the essential precursor to the amino sugar components of the peptidoglycan (PG) and lipopolysaccharide.

respond allosterically to changes in concentrations of the metabolites of glycolysis and thus allow a rapid response to changes in metabolic flux (6, 7).

NagB enzymes have been purified and studied kinetically from a variety of sources: bacteria (2, 3, 8), fungi (9), protozoa (10), and mammals (11, 12). The crystallographic structures of the *E. coli* enzyme and several of its homologues, e.g., enzymes from *Bacillus subtilis* and *Streptococcus mutans* and human isozyme I, have been determined (8, 13–16), and all share the same protein fold and tertiary structure. However, only some of these enzymes are allosteric. The nonallosteric enzymes, e.g., those from *B. subtilis* (8) and *Candida albicans* (9), exist as monomers or dimers. The allosteric enzymes, like *E. coli* NagB ($NagB_{Ec}$), are hexameric.

In archaea and some bacteria, there is a second family of isofunctional but nonhomologous GlcN6P deaminases called NagB-II (17, 18) to distinguish them from the $NagB_{Ec}$ type, now called NagB-I. Their structure is based on the SIS (sugar isomerase) fold, found in the N-terminal domain of GlcN6P synthase (GlmS). At least one NagB-II enzyme is cooperative with respect to its substrate and is allosterically activated by GlcNAc6P (17). This intriguing case of convergent evolution is strong circumstantial evidence that allosteric regulation of NagB is important, at least for certain organisms.

E. coli grows more rapidly on GlcNAc than on GlcN and we previously showed that this was predominantly due to the higher expression of the *nagE-BAC* operon during growth on GlcNAc than on GlcN, i.e., due to transcriptional regulation (19). Growth on GlcNAc produces intracellular GlcNAc6P, which is not only the allosteric activator for NagB but is also the molecular signal causing displacement of NagC from its repressor sites and induction of the *nagE-BAC* genes (20). On the other hand *B. subtilis* grows more rapidly on GlcN than GlcNAc. In this respect *B. subtilis* is unusual compared to other bacilli (21). In addition to the gene encoding the NagB enzyme, which is carried within the *nag* regulon in all bacilli, a second gene (*gamA*) encodes an isozyme of *B. subtilis* NagB ($NagB_{Bs}$) and is part of the *gamR-gamAP* operon. This operon, unique to *B. subtilis* (and a few very close neighbors), is required for the better growth of *B. subtilis* on GlcN. GamA is also responsible for the majority of growth on GlcNAc in this organism (21). As shown below, it is nonallosteric.

We are interested in the biological role of the allosteric activation of NagB in *E. coli* and why an allosteric form has been conserved in some organisms (e.g., *E. coli* and mammals) but is absent in other species such as firmicutes (*B. subtilis*) and fungi (*Candida* and *Aspergillus*). Although we could demonstrate allosteric activation *in vivo* in the case of some mutant forms of $NagB_{Ec}$, there was no evidence that a lack of allosteric activation by GlcNAc6P affected the growth rate of the wild-type strain on GlcN (19). The major effect of growth on GlcNAc was to increase expression of the *nagE-BAC* operon. To uncouple the two effects of GlcNAc6P, allosteric activation of NagB from induction of the *nag* operon via NagC, we put the *nagB* gene under the control of a constitutive promoter and adjusted the amount of the enzyme by changing the translational efficiency of the gene. In this case there was no effect of GlcNAc6P on the quantity of the deaminase, but we still could not show any contribution of the allosteric activation of wild-type *E. coli* NagB to growth on either GlcN or GlcNAc. In fact, the enzyme behaved as if it was fully active during growth on either sugar (22).

High enzymatic activity of NagB during growth on GlcN was an unexpected result, since it seemed unlikely that the intracellular concentration of GlcN6P could attain the concentrations required to saturate the enzyme with substrate. In the absence of the allosteric activator, the enzyme shows sigmoidal velocity versus substrate curves with a half maximal reaction rate ($K_{0.5}$) at 5.5 ± 0.2 mM. The allosteric effector, GlcNAc6P, activates the enzyme by decreasing $K_{0.5}$, which approaches the limit value of 0.75 mM (23, 24). The allosteric kinetics of $NagB_{Ec}$ can be accurately described within the framework of the allosteric symmetry model of Monod, Wyman, and Changeux (the MWC model) (2, 25–28). The allosteric activator GlcNAc6P binds exclusively to the R conformer with a microscopic dissociation constant of 0.035 ± 0.005 mM (values obtained at pH 7.7 and 30°C) (26, 29). GlcNAc6P binding occurs in a range of concentrations 2 orders of magnitude lower than that required for effective saturation of the enzyme by the substrate, GlcN6P.

The observation that NagB appeared to be fully activated, during growth on GlcN, suggested that some GlcNAc6P was also being synthesized. The *nagE-BA* genes, controlled by the GlcNAc6P-sensitive NagC repressor, are partially induced by growth on GlcN (22, 30), This low-level induction supports the idea that some GlcNAc6P is formed during growth on GlcN but also implies that it is only present in a low concentration.

In a first approach, using a liquid chromatography mass spectrometry (LC-MS) method (31, 32), we have measured the effects of growth on GlcN and GlcNAc on the pools of amino sugar phosphates and other glycolytic metabolites to investigate whether the metabolite levels truly reflect the effects on regulation of NagB and NagC previously observed. Growth of *E. coli* on GlcN was found to produce only a small increase in intracellular GlcNAc6P but a very large increase in GlcN6P levels, which together are sufficient to

TABLE 1 Bacterial strains used[a]

Strain	Genotype	Source or reference
MC4100	λ⁻ e14⁻ *araD139* Δ(*arg-lac*)*169 flhD5301 deoC1 relA1 rbsR22 rpsL150* Δ(*fruK-yeiR*) Δ(*fimB-fimE*)*632*	Lab stock (49)
LAA32	MC4100 Δ*nagB*::*cat-sacB*	19
LAA193	MC4100 *gamA*	This study
LAA195	MC4100 $nagB_{Bs}$	This study
LAA199	MC4100	Wild-type recombinant
MC-B1	MC4100 λRS/*nagB-lacZ*	34
MC-B52	MC-B1 Δ*nagEBACD*::Tc	34
MC-B76c	MC-B1 Δ*nagB*	This study
MC-B174c	MC-B1 Δ*nagA*	34
MC-B362	MC-B1 *gamA*	This study
MC-B364	MC-B1 $nagB_{Bs}$	This study
BL21*Δ*nagEBACD*/pRARE	*ompT hsdS gal dcm rne131* (DE3) Δ*nagEBACD*::Tc/pRARE	This study

[a] MC-B76c carries the Δ*nagB*::FRT kanamycin mutation from the Keio collection (50) and was then cured of the kanamycin cassette by transformation with pCP20 (33). The Δ*nagA* mutation in MC-B174c removes only the 5′ half of the *nagA* gene, leaving the *nagC* promoters intact (34). Δ*nagEBACD*::Tc was introduced into BL21* (51) [previously called BL21(DE3) (*rne131*)] by P1 transduction, and the resulting strain was transformed with the pRARE plasmid from Rosetta(DE3) (Novagen).

account for the high enzyme activity previously deduced for NagB during growth on GlcN.

As a continuation of our study of the role of allosteric regulation of *E. coli* NagB, we made chimeric bacteria, where the *E. coli nagB* gene was precisely replaced by the two nonallosteric *nagB* homologues from *B. subtilis*. Using these constructions we have investigated the effect of the absence of allostery on the composition of amino sugar pools and on amino sugar utilization in *E. coli*. The absence of allosteric regulation was found to have surprisingly little impact on bacterial physiology, either during growth on amino sugars or on glucose, at least under the laboratory conditions used.

MATERIALS AND METHODS

Bacteriological methods. (i) Construction of *E. coli* strains with replacements of $nagB_{Ec}$ with $nagB_{Bs}$ and *gamA*. The replacement of *nagB* with a *cat-sacB* cassette was described previously (19). The *nagB*::*cat-sacB* allele was introduced into MC4100 to give strain LAA32. Using the oligonucleotides shown in Table S1 in the supplemental material, DNA fragments carrying *gamA* and *B. subtilis nagB* ($nagB_{Bs}$) genes with 50-bp extensions corresponding to the flanking sequences of *E. coli nagB* ($nagB_{Ec}$) were amplified from *B. subtilis* chromosomal DNA using Advantage HD (Clontech). The purified DNA fragments were used for allelic replacement by the method of Datsenko and Warner (33) using LAA32 carrying pKD46 as a recipient. Loss of the *cat-sacB* cassette was selected by sucrose resistance and screening for chloramphenicol sensitivity. Sucrose sensitivity was tested on BYE plates (10 g/liter Bacto-tryptone, 5 g/liter yeast extract, 40 g/liter sucrose). Candidate bacteria were purified, and correct allelic replacements were verified by sequencing a 1.3-kb PCR fragment, Nag14-Nag53, encompassing the *nagB* chromosomal locus of *E. coli*. Bacterial strains are listed in Table 1.

Growth rates were measured in minimal morpholinepropanesulfonic acid (MOPS) medium supplemented with 10 mM glucose, GlcN, or GlcNAc at 37°C as described previously (19). The *gamA* and $nagB_{Bs}$ alleles were also introduced by P1 transduction into MC-B52 (a Δ*nagEBACD*::Tc strain carrying a *nagB-lacZ* fusion on a λ lysogen) (22), with selection for growth on GlcNAc and screening for tetracycline sensitivity. The β-galactosidase activities were measured as described previously (22).

(ii) Metabolite extracts and measurements. Samples of bacterial cultures were collected by using a filter method, and metabolites were extracted in hot ethanol as described previously (32). Starting from a fresh bacterial colony on Luria-Bertani (LB) plates, precultures were grown in minimal MOPS medium with 0.4% glycerol as the carbon source overnight at 37°C. Bacteria were diluted into minimal MOPS medium with 10 mM glucose, GlcN, or GlcNAc as carbon source at an optical density (A_{650}) of 0.02 and grown with vigorous shaking at 37°C. At an A_{650} of 0.5, three 2-ml aliquots were rapidly removed, filtered on polyvinylidene difluoride membranes (Durapore; 0.45-μm pore size, 2.5-cm diameter), washed with 4 ml of the same prewarmed medium at 37°C, and rapidly transferred to 4 ml of 60% ethanol prewarmed at 78°C. An aliquot of uniformly ^{13}C-labeled metabolite extract, prepared as described below, was immediately added. The filters were held at 78°C for 3 min, agitated occasionally, and then transferred to ice. The ethanolic solution of bacteria was centrifuged to remove bacterial debris and the supernatants stored at −80°C prior to drying by speed evaporation. The dried pellets were stored at −80°C until analysis. Metabolites were detected using an ion-pairing ultrahigh-performance liquid chromatography-tandem mass spectrometry method as described previously (31, 32). Dry metabolite extracts were resuspended in 100 μl of water, 10 μl of which were injected on a Waters Acquity UPLC apparatus with a Waters Acquity T3 end-capped reversed-phase column (150 by 2.1 mm by 1.8 μm; Waters Corporation, Milford, MA). The UPLC system was coupled to a triple-quadrupole mass spectrometer (TSQ Quantum Triple Quadrupole with ElectroSpray Ionization; Thermo Scientific, Waltham, MA) for metabolite detection. Three filters were analyzed for each culture, and two independent, biological replicate, cultures were analyzed.

Uniformly [^{13}C]glucose-labeled metabolites for an internal standard were made from an overnight microanaerobic culture of bacteria growing in minimal MOPS medium in which the only carbon source was 0.1% [^{13}C]glucose. Bacteria were filtered, and metabolites were extracted as described above by filtering 20 ml of bacteria at an optical density at 650 nm (OD_{650}) of 0.5 on 5.0-cm-diameter filters and extracting with 6 ml of 60% ethanol at 78°C. The dried pellets were resuspended so that bacteria were at an optical density of ~1 per 0.1 ml of water. Labeled extracts were made from MC-BI (wild type) and MC-B174C (*nagA* mutant) in which GlcNAc6P accumulates (34). The two ^{13}C-labeled extracts were mixed to ensure the presence of both labeled GlcN6P and GlcNAc6P and stored in aliquots at −20°C. The calculated concentrations of metabolites in extracts were normalized to intracellular concentrations using the preestablished conversion factors, 0.5 mg/OD unit of bacteria and a specific cell volume of 2 μl/mg (32, 35).

(iii) Overproduction, purification, and kinetic properties of GlcN6P deaminases. The $nagB_{Bs}$ gene was obtained by total synthesis (Epoch Laboratories, Missouri City, TX) and subcloned into the T7 expression vector pET22b+, which was used to transform the *E. coli* strain BL21*Δ*nagEBACD*/pRARE. The *gamA* gene was obtained by PCR using

the oligonucleotides GamA2NdeI and GamA3BamHI (see Table S1 in the supplemental material) using chromosomal DNA from *B. subtilis* strain SSB1002 (21). The PCR product was inserted into the T7 expression vector pJES307 and also transformed into BL21*Δ*nagEBACD*/pRARE.

The transformed cells were grown in LB broth at 30°C, and enzyme expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 6 h before harvesting. The cells were disrupted by mild sonication, and the extract was centrifuged at 45,000 × *g* for 30 min. Both deaminases were purified by preparative column chromatography using the same procedure. The clarified extract was applied to a Source 15Q anion-exchange preparative column equilibrated at pH 7.8 at 25°C and eluted with a gradient of NaCl from 0 to 1 M prepared in 50 mM Tris-HCl buffer at the same pH. $NagB_{Bs}$ eluted at 0.35 M NaCl, whereas GamA appeared in the eluate at a 0.5 M salt concentration. To remove the remaining impurities, each preparation was refined by molecular sieving in a Superdex S-75 column run in the same buffer solution. In both preparations a single peak of electrophoretically pure protein was obtained. *E. coli* NagB was purified by allosteric site affinity chromatography as described previously (2).

Kinetic studies. GlcN6P deamination rate was measured at 37°C in 100 mM Tris buffer (pH 7.4), with the concentrations of GlcN6P and GlcNAc6P indicated in figures. The amount of Fru6P formed at a fixed time was measured using the previously described procedure (2), keeping the reaction below 5% conversion of the initial substrate concentration. The kinetic mechanism of the $NagB_{Ec}$-catalyzed reaction is "rapid equilibrium random" (36). This allows equating *v*/*V* data (fractions of V_{max} measured as initial velocity divided by maximum velocity) from kinetic experiments to fractions of substrate saturation ($\bar{y}_S$) and K_m values as true dissociation constants.

Data analysis. Data fitting and graph plotting were performed with Prism 6.0 for Mac OS X (GraphPad Software, Inc., USA). Nonlinear regression analyses were always checked by inspection of their residual plots. Errors given in the tables and text are the standard deviations of the regressions.

RESULTS

Effect of growth on amino sugars on intracellular pools of GlcN6P, GlcAc6P and downstream metabolism. Wild-type *E. coli* (MC-B1) was grown in minimal medium with glucose, GlcN, or GlcNAc as a carbon source, and metabolites were extracted and analyzed by targeted liquid chromatography-mass spectrometry (32) for a set of common glycolytic metabolites to measure the impact of growth on GlcN and GlcNAc on bacterial physiology (Fig. 2, strain 1). As expected, growth on GlcN or GlcNAc produced major changes in the concentrations of the amino-sugar phosphates (Fig. 3; see Table S2 in the supplemental material) but relatively small differences in the concentrations of compounds of lower glycolysis and the tricarboxylic acid (TCA) cycle. During growth on glucose very little GlcNAc6P was detected (14 ± 6 μM). The basal level of GlcN6P was ~5-fold higher (62 ± 14 μM). GlcN6P, an essential starting material for synthesis of the peptidoglycan (PG) of cell walls and lipopolysaccharide (LPS) of the outer membrane, is synthesized by the enzyme glucosamine-6P synthase (GlmS) (Fig. 1B). However, the only known source of GlcNAc6P in the cell, in the absence of exogenous amino sugars, is from PG recycling (34, 37). During growth on GlcN, the level of GlcN6P increased over 100-fold (to about 9 mM) (Fig. 3B; see Table S2 in the supplemental material). The level of GlcNAc6P, on the other hand, increased a maximum of 2-fold. During growth on GlcNAc the concentrations of GlcN6P and GlcNAc6P increased about 10- and 20-fold, respectively (Fig. 3C; see Table S2 in the supplemental material).

During growth on glucose the concentration of glucose-6P (G6P) was higher than during growth on the amino sugars, but only by ~2-fold. The concentrations of fructose-6P (F6P), the first common glycolytic intermediate from the metabolism of the three sugars, and most of the compounds of lower glycolysis and the TCA cycle were essentially unchanged in all three media (Fig. 2). However, the concentration of fructose 1,6-bis-phosphate (FBP) was ~3-fold lower during growth on GlcN than on glucose or GlcNAc. Dihydroxyacetone phosphate (DHAP) and citrate/isocitrate were also lower during growth on GlcN (Fig. 2), but, overall, the glycolytic pathway was not affected by the change in carbon source.

Effect of deletions in *nagB* and *nagA* on amino sugar metabolic pools. Deletion of the genes for GlcN6P deaminase (*nagB*) and GlcNAc6P deacetylase (*nagA*) prevents the use of GlcNAc as a carbon source. The Δ*nagA* mutation is known to provoke the accumulation of high concentrations of GlcNAc6P from interruption of the PG recycling pathway (34, 37, 38). The liquid chromatography-mass spectrometry metabolic measurements confirmed these previous results, determining an intracellular concentration of ~2 mM GlcNAc6P within the *E. coli* Δ*nagA* cells growing on glucose (Fig. 3A; see Table S2 in the supplemental material).

GlcN6P was at very low levels in the *nagA* strain (see Table S2 in the supplemental material). This is certainly due to two related but independent causes. The accumulation of GlcNAc6P in the Δ*nagA* mutant produces induction of NagC-repressed genes, resulting in large overproduction of the proteins of the *nag* operon, including NagB (19, 20). The consequently high levels of NagB will be fully active, because of the high concentration of its allosteric activator, GlcNAc6P, and will convert any available GlcN6P to F6P. Second, since NagA is an integral part of the PG recycling pathway (37), the *nagA* mutation prevents all PG recycling, so that no GlcN6P is regenerated from PG degradation, and all amino sugar containing cell wall components must be made from GlcN6P synthesized *de novo* by GlmS.

On the other hand, deletion of the *nagB* gene had no strong effect on the amino sugar pools. In particular, there was very little increase in the GlcN6P concentration (at most 30%), implying that NagB is not usually acting on the GlcN6P pool and/or is not affecting regulation of GlcN6P homeostasis. Since GlcN6P synthesized by GlmS during growth on glucose is the essential precursor of all cell wall PG and LPS amino sugar components, it is necessary to protect newly synthesized GlcN6P from degradation by NagB. This could be achieved by controlling GlmS activity (39, 40).

Activity of $NagB_{Ec}$ *in vitro* with physiological concentrations of GlcNAc6P and GlcN6P. We performed *in vitro* enzyme assays using purified *E. coli* GlcN6P deaminase to investigate the effect of GlcN6P and GlcNAc6P, over the concentration ranges measured *in vivo*, on the kinetic parameters and activity of the enzyme. Figure 4A shows the kinetic pattern of velocity versus substrate concentration at pH 7.4 and 37°C. The data were fitted to the general MWC allosteric equation (25). The fitted parameter for GlcNAc6P binding was 66 ± 17 μM, which is somewhat higher than our previously reported values at pH 7.7 and 30°C by direct binding studies (36 ± 3 μM [41]) or from kinetic measurements (35 ± 5 μM [26]). The measured intracellular concentration of GlcNAc6P during growth on GlcN is less than this (25 ± 7 μM; Fig. 3; see Table S2 in the supplemental material). The area within the gray lines on Fig. 4A covers the substrate concentrations 5 to 12 mM GlcN6P with 10 to 30 μM for the allosteric activator,

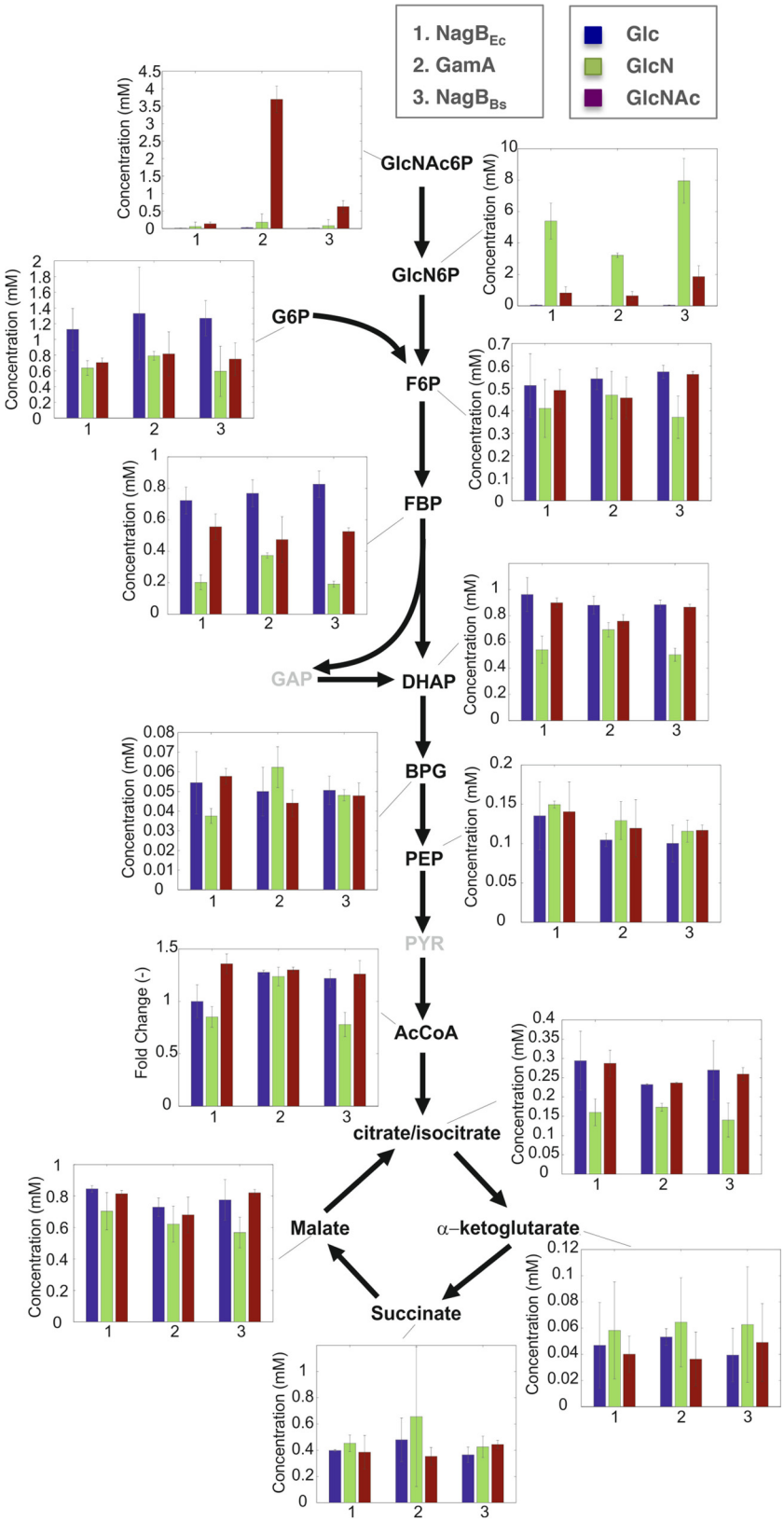


FIG 2 Effect of growth on amino sugars on glycolysis. A schematic view of glycolysis and the TCA cycle during growth on Glc, GlcN, or GlcNAc is shown. The intracellular concentrations of the compounds indicated are shown for the three strains: MC-B1 expressing wild-type $NagB_{Ec}$ (strain 1), MC-B362 expressing GamA (strain 2), and MC-B364 expressing $NagB_{Bs}$ (strain 3). The concentrations of metabolites during growth on glucose (Glc) are shown in blue, those on GlcN are shown in green, and those on GlcNAc are shown in brown/red. Glyceraldehyde-3P (GAP) and pyruvate (PYR) are unstable during the extraction process and were not quantified. The complete analysis was repeated twice. A representative experiment is shown with means and standard deviations of three replicate filters. Data for GlcN6P and GlcNAc6P are expanded in Fig. 3.

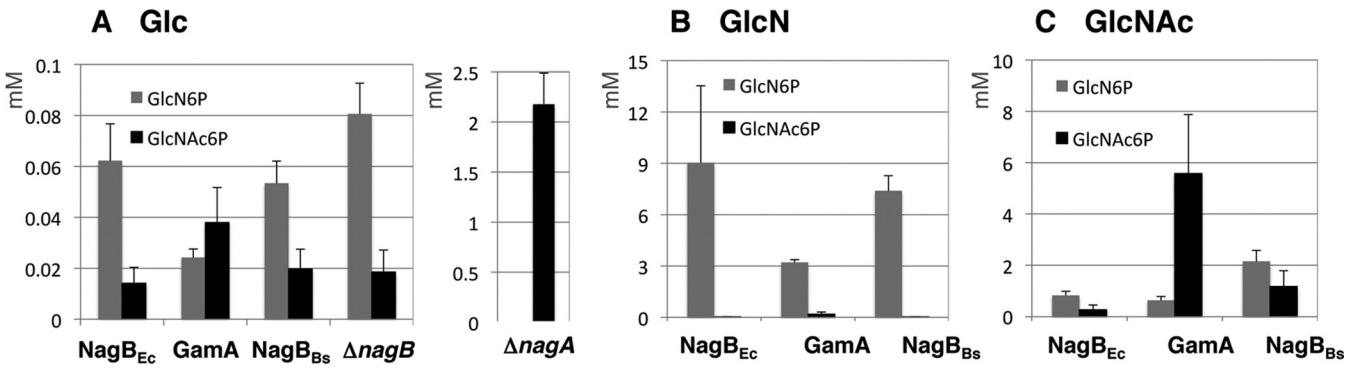


FIG 3 Effect of growth on glucose (Glc), GlcN, and GlcNAc on the pools of GlcN6P and GlcNAcP in the wild type, in *nagB* recombinants expressing the *B. subtilis* GlcN6P deaminases, and in strains with the *nagA* and *nagB* genes deleted. Intracellular concentrations of GlcN6P and GlcNAc6P during growth of wild-type *E. coli* NagB$_{Ec}$ (MC-B1), *E. coli* expressing GamA (MC-B362), and *E. coli* expressing NagB$_{Bs}$ (MC-B364) in Glc (A), GlcN (B), and GlcNAc (C) and the Δ*nagA* (MC-B174c) and Δ*nagB* (MC-B76c) deletion strains in glucose are shown. The results are means with the standard deviations for two biological replicate cultures (six filters). Note the change in scales for concentrations. The same data are given in numerical form in Table S2 in the supplemental material.

GlcNAc6P, i.e., the range of concentrations measured during growth on GlcN. The enzyme under these conditions is operating at between 50 and 90% of its maximum rate. These values are consistent with the high NagB activity deduced for the enzyme during growth on GlcN (22).

Characterization of the NagB isozymes from *B. subtilis*. The NagB enzyme of *B. subtilis* has been purified, and its crystallographic structure was determined previously (8), but no information was available in the literature for the GamA enzyme. Both GamA and the NagB$_{Bs}$ enzymes were purified, and their reaction kinetics were compared to those of NagB$_{Ec}$ under identical conditions. NagB$_{Bs}$ and GamA were both confirmed to be nonallosteric and were unaffected by the presence of GlcNAc6P (Fig. 5A). Their catalytic activities were compared to that of NagB$_{Ec}$ in its activated form, i.e., saturated with GlcNAc6P (Fig. 5B). The K_m for GlcN6P for NagB$_{Bs}$ and GamA were nearly 2- and 4-fold higher than NagB$_{Ec}$ (in its high-affinity R allosteric state) while the k_{cat} values for the two *B. subtilis* enzymes were ~2-fold lower than their *E. coli* counterpart (Fig. 5B). These differences are reflected in the lower specificity constants (also called catalytic efficiency) for the two *B. subtilis* enzymes.

Growth of *E. coli* with *nagB*$_{Ec}$ replaced by nonallosteric GlcN6P deaminase genes of *B. subtilis*. In an attempt to assess the impact that NagB$_{Ec}$ allostery has on growth of *E. coli* on amino

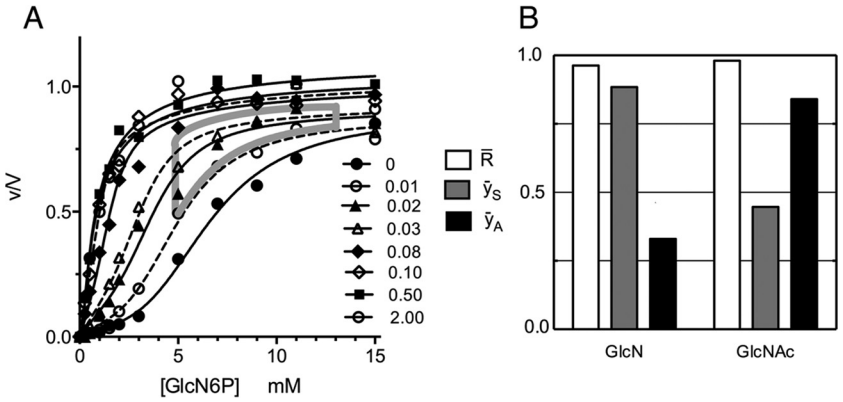
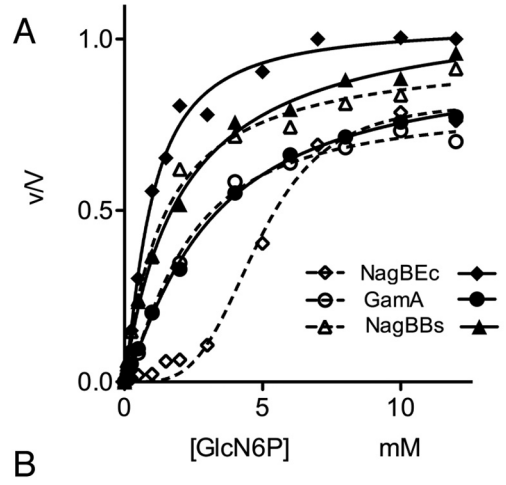


FIG 4 Activation of NagB$_{Ec}$ by subsaturating amounts of its allosteric activator, GlcNAc6P. (A) Enzyme activity expressed as *v/V* (fraction of V_{max} measured as initial velocity divided by maximum velocity) was plotted versus the GlcN6P concentration at the GlcNAc6P concentrations shown. The *v/V* ratio is equivalent to the fraction of substrate saturation, $\bar{y}_S$, according to the known kinetic mechanism of NagB$_{Ec}$. Assays were performed at 37°C and pH 7.4, conditions chosen to resemble the conditions of the bacterial cultures (Fig. 2, 3, and 6). The area bordered by a gray line indicates the range in GlcN6P and GlcNAc6P concentrations measured *in vivo* during growth on GlcN, where allosteric activation can be expected to be significant. The NagB$_{Ec}$ concentration was 5 nM (hexamer). The data were fitted by nonlinear global regression analysis using the MWC equation for an exclusive-binding allosteric activator (25). The fitted MWC parameters are $K_{R(GlcN6P)} = 0.85 \pm 0.07$ mM, $K_{A(GlcNAc6P)} = 66 \pm 17$ μM, L_0 (MWC allosteric constant, defined by the T_0/R_0 ratio) $= 5 \times 10^4$, and c (defined as K_R/K_T) $= 0.069 \pm 0.005$. These values can be compared to the previously measured values at 30°C and pH 7.7 [$K_{R(GlcN6P)} = 0.55 + 0.05$ mM, $K_{A(GlcNAc6P)} = 35 \pm 5$ μM, $L_0 = 10^6$, and $c = 0.025 \pm 0.002$] (24, 26). Note that $K_{A(GlcNAc6P)}$ is nearly 2-fold higher, and the parameter c, related to the free energy change of the *T-R* transition (K_R/K_T), is also 2-fold higher whereas L_0, the *T/R* ratio of the ligand-free enzyme, is 20-fold lower than at 30°C. (B) Fraction of NagB$_{Ec}$ in the R conformer ($\bar{R}$) and fractional saturation of the enzyme with substrate ($\bar{y}_S$) or allosteric activator ($\bar{y}_A$) during growth on GlcN and GlcNAc. $\bar{R}$ was calculated with the parameters given in panel A using the following state equation: $\bar{R} = [(1 + S/K_S)^6]/\{[L_0/(1 + A/K_A)^6](1 + cS/K_S)^6 + (1 + S/K_S)^6\}$ (from reference 52, where S and A are the concentrations of substrate [GlcN6P] and allosteric activator [GlcNAc6P] and K_S and K_A are their microscopic dissociation constants) and the measured metabolite concentrations (Fig. 3; see Table S2 in the supplemental material). The fraction of the enzyme active sites occupied by GlcN6P ($\bar{y}_S$) is the *v/V* value (see above). The fraction of allosteric sites occupied by GlcNAc6P ($\bar{y}_A$) was calculated as [GlcNAc6P]/(K_A + [GlcNAc6P]). This is valid because only the R conformer of NagB$_{Ec}$ binds the allosteric effector, GlcNAc6P.



	K_M (GlcN6P)	k_{cat}	Catalytic efficiency k_{cat}/K_M
	mM	s^{-1}	s^{-1} mM^{-1}
$NagB_{Ec}$	0.85 ± 0.5	220 ± 10	259
GamA	3.5 ± 0.5	92 ± 5	26
$NagB_{Bs}$	1.5 ± 0,3	110 ± 10	73

FIG 5 (A) Kinetic properties of three GlcN6P deaminases, $NagB_{Ec}$, GamA, and $NagB_{Bs}$. The enzyme activity, expressed as *v/V*, of $NagB_{Ec}$, GamA, and $NagB_{Bs}$ (and equivalent to the substrate saturation fraction [$\bar{y}$]), was measured in the absence (dotted lines) or presence (solid lines) of 1 mM GlcNAc6P at 37°C and pH 7.4. Data were fitted to the hyperbolic equation except for $NagB_{Ec}$ in the absence of GlcNAc6P, which was fitted to the MWC equation for allosteric activation (2, 25). Enzyme concentrations were normalized to 40 nM per subunit. GlcNAc6P has no effect on the activities of the *B. subtilis* enzymes under the conditions used. (B) Kinetic parameters for the three enzymes in the presence of GlcNAc6P. Catalytic efficiency (also known as the specificity constant) was calculated as k_{cat}/K_m. Substrate inhibition was previously reported for $NagB_{Bs}$, when measured at pH 8.0 and at 22°C (8). Using our assay conditions (pH 7.4 and 37°C), we did not detect any inhibition by GlcN6P (up to 10 mM) or by GlcNAc6P (1 mM), but substrate inhibition was observed with our preparation when assayed under the conditions described previously (8).

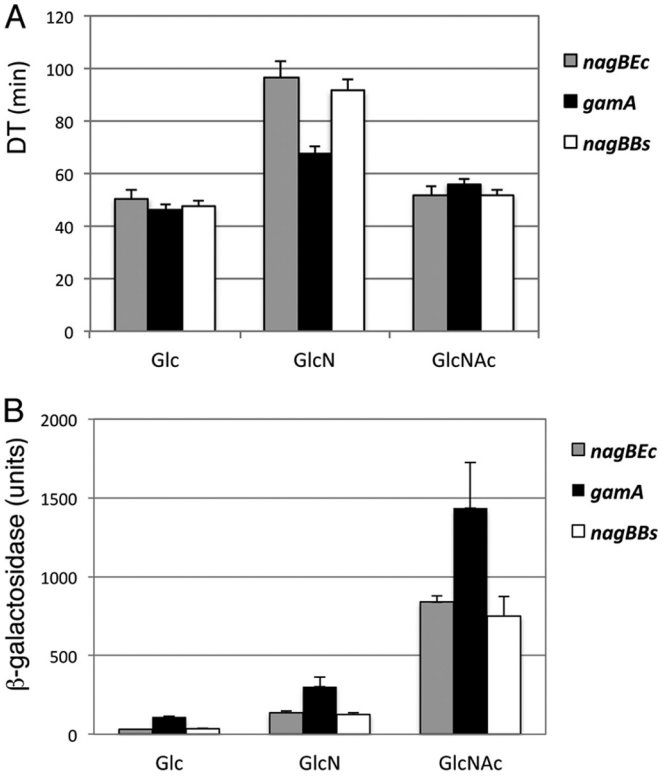


FIG 6 (A) Growth rates of wild-type *E. coli* and *E. coli* with the $nagB_{Bs}$ and *gamA* replacements on Glc, GlcN, and GlcNAc. Bacterial strains LAA199 ($nagB_{Ec}^+$), LAA193 ($gamA^+$), and LAA195 ($nagB_{Bs}^+$) were grown in minimal MOPS medium at 37°C with 10 mM Glc, GlcN, or GlcNAc as the carbon source. Doubling times (DT) were calculated in exponential growth phase and are expressed in minutes ± the standard deviations for six biological replicate cultures. (Similar DT were measured for the strains carrying the *nagB-lacZ* fusion on a λ lysogen, MC-B1, MC-B362, and MC-B364.) (B) Transcriptional regulation by NagC of a *nagB-lacZ* fusion (on a λ lysogen) in wild-type *E. coli* and in *E. coli* with the $nagB_{Bs}$ and *gamA* replacements, during growth on Glc, GlcN, and GlcNAc. MC-B1 ($nagB_{Ec}^+$), MC-B362 ($gamA^+$), and MC-B364 ($nagB_{Bs}^+$) β-galactosidase activities (Miller units) were measured in minimal MOPS medium at 37°C with 10 mM Glc, GlcN, or GlcNAc as the carbon source.

sugars and on amino sugar homeostasis, we exactly replaced the native *E. coli nagB* gene ($nagB_{Ec}$) with the genes for the two *B. subtilis* GlcN6P deaminase isozymes ($nagB_{Bs}$ and *gamA*). Although both $NagB_{Bs}$ and GamA had higher K_m and lower k_{cat} values than $NagB_{Ec}$ (when saturated with GlcNAc6P) *in vitro*, the replacement of the $nagB_{Ec}$ open reading frame with $nagB_{Bs}$ produced a strain that grew rather similarly to bacteria with the wild-type *E. coli* enzyme on glucose (Glc), GlcN, and GlcNAc (Fig. 6A). The GamA strain also grew similarly to the wild-type *E. coli* strain on glucose and GlcNAc, but it grew significantly faster on GlcN (Fig. 6A). These observations show that the absence of allosteric regulation of NagB does not impair growth on GlcNAc. However, the improvement in growth of the *gamA* strain on GlcN was interesting, especially since GamA is responsible for the higher growth rate of *B. subtilis* on GlcN compared to GlcNAc (21).

Amino sugar metabolite levels in bacteria with heterologous *B. subtilis* GlcN6P deaminase genes. Metabolites of the GamA and $NagB_{Bs}$ recombinants during growth on glucose, GlcN, and GlcNAc were measured in parallel with the wild-type $NagB_{Ec}$ strain (Fig. 2, strains 2 and 3). Remarkably, GlcN6P and GlcNAc6P, as well as most of the downstream glycolytic metabolites in the $NagB_{Bs}$ recombinant strain, were similar to those in the wild-type, $NagB_{Ec}$, strain during growth on glucose and GlcN (compare strains 3 and 1, in Fig. 2). However, GlcN6P and GlcNAc6P levels were about 2- and 4-fold higher, respectively, in the $NagB_{Bs}$ strain during growth on GlcNAc (Fig. 2 and 3; see Table S2 in the supplemental material), even though the growth rates are comparable.

The presence of the *gamA* allele (strain 2, in Fig. 2) had major effects on the amino sugar pools in all three media. In particular, the levels of GlcN6P were ~2-fold lower in the GamA strain compared to those with *E. coli* or *B. subtilis* NagB, while the GlcNAc6P concentrations were strongly increased in all three media (Fig. 3; see Table S2 in the supplemental material). The GlcNAc6P concentrations were 2-fold higher on glucose, ~7-fold higher on GlcN, and nearly 20-fold higher during growth on GlcNAc. However, there were no significant effects on the downstream glycolytic intermediates in the GamA strain compared to $NagB_{Ec}$ or $NagB_{Bs}$ strains, except for a small effect on FBP and DHAP. Confirmation that the GlcNAc6P levels were significantly higher in the GamA strain came from measurements of the NagC repression of a *nagB-lacZ* fusion (Fig. 6B). Expression from the *nagB* promoter is 2-fold higher in the GamA strain than in the wild-type $NagB_{Ec}$ or $NagB_{Bs}$ recombinant.

In the GamA recombinant, NagC is controlling expression of the *gamA* gene (from the *nagB* promoter in *cis*), as well as the *nagB-lacZ* fusion (located in *trans* on a lambda lysogen). A higher GlcNAc6P concentration, in the GamA recombinant would lead to less NagC repression of the *gamA* gene, and hence more GlcN6P deaminase activity, and would account for the higher growth rate on GlcN. This scenario was supported by measurements of the intracellular levels of the three deaminases and NagA (see Fig. S1 in the supplemental material). Effectively, the relative level of GamA was ca. 50% higher in the GamA replacement strain than either $NagB_{Bs}$ or $NagB_{Ec}$ strains during growth on GlcN (see Fig. S1 in the supplemental material). On the other hand, the NagA protein level was much lower in the *gamA* strain than in the strains with the other two *nagB* alleles (see Fig. S1 in the supplemental material). A smaller amount of NagA protein (and hence GlcNAc6P deacetylase activity) would account for the higher concentration of its substrate, GlcNAc6P. We conclude that the replacement of the $nagB_{Ec}$ gene with the *B. subtilis gamA* gene has introduced a polarity into expression of the *nagBA* genes, resulting in lower *nagA* expression and hence less GlcNAc6P deacetylase activity and higher GlcNAc6P levels (see Fig. S2 in the supplemental material for more details). The nature of the signal provoking the polarity in the *gamA* gene has not been investigated.

These unexpected transcriptional effects, produced by the replacement of the $nagB_{Ec}$ gene with the *gamA* gene, meant that this strain was not suitable to study the effects of loss of allosteric regulation on growth of *E. coli*. We thus focused our attention on the $nagB_{Bs}$ replacement. As shown in Fig. 2, 3, and 6, the loss of allostery in the $NagB_{Bs}$ recombinant has only minor consequences on the amino sugar metabolite pools and downstream glycolytic metabolites and no appreciable effect on the growth rates.

DISCUSSION

Effect of growth on amino sugars on the *E. coli* metabolome. Growth on GlcN or GlcNAc produced large increases in the concentrations of GlcN6P or GlcNAc6P (Fig. 2 and 3; see Table S2 in the supplemental material). However, the glucose-6P (G6P) concentration was not much higher during growth on glucose than on GlcN or GlcNAc, presumably due to its synthesis by the gluconeogenesis pathway and reflecting its pleiotropic roles in cell metabolism and glycosyltransferase reactions. The concentration of F6P, and most downstream glycolytic intermediates, was rather constant in all media for the three strains. The main exception was the concentration of fructose-1,6-bis-phosphate (FBP), which was slightly lower on GlcNAc than on glucose and 2- to 3-fold lower on GlcN. FBP has been described as a metabolic flux sensor (4) and so its lower concentration reflects the lower growth rate on GlcN, compared to glucose or GlcNAc (Fig. 6A). It is interesting that in the GamA recombinant, which does grow more rapidly on GlcN than the $NagB_{Ec}$ or $NagB_{Bs}$ strains, the FBP concentration was only 2-fold lower than on glucose, whereas in the $NagB_{Ec}$ and $NagB_{Bs}$ strains the FBP was 3-fold lower, corroborating its role in metabolic flux monitoring (Fig. 2).

Intracellular pools of amino sugar phosphates and allosteric regulation of *E. coli* GlcN6P deaminase. The high intracellular pool of GlcN6P, measured during growth on GlcN, explains why $NagB_{Ec}$ appeared in its high activity R state in our previous growth tests in GlcN (22). Using the measured GlcN6P and GlcNAc6P concentrations during growth on glucose, GlcN, and GlcNAc, together with the MWC allosteric parameters, derived from the data of Fig. 4A, we can calculate the allosteric status and occupancy of the active and allosteric sites of the enzyme, during growth on GlcN and GlcNAc (Fig. 4B). The fraction of enzyme in the high-affinity R conformer ($\bar{R}$) is close to unity (0.96 to 0.98) in both GlcN and GlcNAc media. The fraction of enzyme saturated with substrate ($\bar{y}_S$) is also very high (0.88) in GlcN but much lower (0.45) in GlcNAc. On the other hand the fraction of enzyme with bound GlcNAc6P ($\bar{y}_A$) is high in GlcNAc (0.84) but much lower during growth on GlcN (0.33).0 The measured intracellular GlcNAc6P concentration during growth on GlcN (25 $\pm$ 10 μM) is lower than the K_A value for GlcNAc6P (66 $\pm$ 17 μM), accounting for the partial occupancy. Thus, we can see that during growth on GlcN the high enzyme activity is driven by positive cooperative binding of the substrate, GlcN6P, producing the high $\bar{y}_S$ and $\bar{R}$ values. On the other hand, during growth on GlcNAc, it is the binding of the allosteric activator, GlcNAc6P, which shifts the enzyme to its R conformer with higher substrate affinity (high $\bar{R}$ value) but with a much lower substrate occupancy ($\bar{y}_S$), due to the lower GlcN6P concentration (Fig. 4B). During growth in glucose, the intracellular concentrations of both amino sugar-phosphates are so low that the small amount of $NagB_{Ec}$ present is almost all in the ligand-free T state.

Intracellular pool of GlcNAc6P and induction of the *nag* regulon. The partial induction of the NagC repressed *nagB* genes during growth on GlcN, as measured by the $nagB_{Ec}$-*lacZ* fusion in *trans* (Fig. 6B), implies that the concentration of GlcNAc6P present during growth on GlcN is insufficient for complete displacement of the NagC repressor from the *nagE-B* operators. Interestingly, the measured GlcNAc6P concentrations during growth of the GamA recombinant on glucose and GlcN are higher than in the other strains, which can account for the higher induction of *nagB* promoter activity and increased GamA protein level measured in this strain (Fig. 3 and 6B; see also Table S2 and Fig. S1 in the supplemental material). Even during growth on GlcNAc, expression of the *nagB-lacZ* fusion was 2-fold higher in the GamA strain than in the $NagB_{Ec}$ strain, implying that the concentration of GlcNAc6P in the $NagB_{Ec}$ strain during growth on GlcNAc was not leading to complete loss of NagC repression.

NagB allostery and GlcN6P homeostasis. GlcN6P is the key starting compound for synthesis of all the amino sugar containing constituents of the cell wall and outer membrane. It might be expected that this pool should be protected from a cytoplasmic catabolic enzyme. One possible function of the allosteric regulation of $NagB_{Ec}$ could be to maintain the enzyme in its state with low substrate affinity (T state), during growth in the absence of exogenous amino sugars. The measured intracellular concentrations of *de novo*-synthesized GlcN6P and of GlcNAc6P (produced by peptidoglycan recycling) during growth on glucose, are both below the concentrations required to displace the conformational equilibrium of $NagB_{Ec}$ toward the active R state (Fig. 4A). However, the absence of allosteric regulation in the $NagB_{Bs}$ recombinant had apparently no, or only a minor effect, on the basal level of GlcN6P levels (maximum 20% decrease; see Table S2 in the supplemental material). Indeed, even in the *nagB* deletion strain, the intracellular GlcN6P concentration did not change very much (30% increase; see Table S2 in the supplemental material). Thus, the absence of a GlcN6P deaminase or the presence of a constitutively active version did not significantly affect the GlcN6P levels. Amino sugar homeostasis is achieved by a sophisticated posttranscriptional regulation of the expression of the *glmS* gene, encoding

GlcN6P synthase. At high GlcN6P concentrations, expression of *glmS* is low, but when the GlcN6P concentration falls, a regulatory cascade is initiated, resulting in higher GlmS levels (40, 43). This mechanism must maintain GlcN6P levels despite a potentially active deaminase (39). How the level of GlcN6P is monitored by the bacteria and how regulation of GlcN6P synthase and deaminase affects nitrogen metabolism are currently unknown.

We attempted to determine whether the *glmS* regulatory loop was activated in the $nagB_{Bs}$ strain. We looked for evidence of any loss of GlcN6P by futile cycling by performing a growth yield experiment, using limiting amounts of glucose or GlcNAc as carbon source, and the three strains carrying *nagB* isozymes. All grew at the same rate and stopped growing at the same optical density (data not shown), and so any futile cycle resulting in loss of energy from converting glutamine to ammonia cannot be seen with this simple technique. We also carried out competitive growth experiments between the *gamA* or $nagB_{Bs}$ recombinants and wild-type $nagB_{Ec}$ strains (44, 45). These experiments confirmed the growth advantage of the GamA strain in GlcN (fitness = 1.37 ± 0.16) and also suggested a small gain of fitness of the $NagB_{Bs}$ strain in GlcN (1.13 ± 0.14). However, we detected no significant change in fitness in glucose, even after 40 generations, for either of the recombinant strains, compared to the wild-type $nagB_{Ec}$ strain, or indeed compared to a strain carrying a *nagB* deletion. So, under our laboratory conditions, there is no evidence that an allosteric NagB enzyme is conferring a fitness advantage (or disadvantage) to the bacteria. The lack of any physiological response to loss of NagB allostery demonstrates the robustness of the amino sugar regulon and that other layers of regulation are involved in ensuring GlcN6P homeostasis. Other strategies could exist which prevent the establishment of a futile cycle involving NagB and GlmS in bacteria. For example, the enzymes of the early steps of cell wall amino sugar components, including GlmS, GlmM and GlmU (Fig. 2), could be part of a specialized bacterial microcompartment (46), which would direct the newly synthesized GlcN6P toward the next step in the biosynthetic pathway and prevent it from being available for immediate degradation by GlcN6P deaminase.

Concluding remarks. GlcNAc6P has dual regulatory functions in *E. coli*, as the allosteric effector of both NagC and NagB. Quantitation of the cellular concentrations of amino sugar-phosphates has allowed us to explain the anomaly that growth on GlcN produces strong activation of the enzyme GlcN6P deaminase ($NagB_{Ec}$) but only very weak induction of the NagC-repressed *nagE-BACD* genes when both $NagB_{Ec}$ and NagC are sensitive to the same allosteric effector, GlcNAc6P. Strong cooperative binding of the substrate GlcN6P mostly drives the high activation of $NagB_{Ec}$, whereas the minimal increase in GlcNAc6P results in just a partial displacement of NagC from its operators. Thus, despite having the same allosteric effector, GlcNAc6P, NagB and NagC are activated to very different levels during growth on GlcN.

In the absence of a suitable nonallosteric *E. coli* GlcN6P deaminase, we have studied two heterologous, authentic, nonallosteric enzymes, within the context of the *E. coli nagE-BACD* operon. The *B. subtilis* genes precisely replaced the *E. coli nagB* open reading frame, so that the heterologous genes are expressed from and are subject to the same transcriptional regulatory signals as the native *E. coli* gene. Indeed regulated induction by growth on GlcN and GlcNAc was verified by Western blotting (see Fig. S1 in the supplemental material). The loss of an allosteric deaminase seemed to have no significant effect on the growth rate or competitive fitness for the $nagB_{Bs}$ recombinant, at least under our laboratory conditions (Fig. 6). (The growth advantage of the GamA recombinant on GlcN was shown to be an artifact due to an indirect transcriptional effect on *nagA* expression and not an effect of the GamA enzyme *per se* [see Fig. S2 in the supplemental material for further details].)

Finally, it must be admitted that we have not identified a role for the allosteric regulation of $NagB_{Ec}$, nor have we determined why it has been maintained through evolution in *E. coli* and in some other species, including humans. The ability to use amino sugars is an important criterion in the infection and colonization of mammalian hosts by both commensal and pathogenic bacteria (47, 48). *E. coli* and other *Enterobacteriaceae* have to endure periods of "feast and famine" as part of a dual life cycle, alternating between habitats rich in nutrients in mammalian hosts, or in aquatic or terrestrial habitats, usually habitats poor in nutrients. Allostery could be advantageous for survival when bacteria are competing in a dynamic environment for a limited amount of nutrients.

ACKNOWLEDGMENTS

This study was supported in France by grants from the CNRS (to UPR9073, renamed UMR8261), by ANR-09-BLAN 0399 GRONAG, and by the Initiative d'Excellence program from the French State (grant ANR-11-LABX-0011-01 DYNAMO) and in Mexico by CONACyT (grants 99857-Q and International Cooperation France Mexico grant 116074 GRONAG) and by UNAM through the programs DGAPA-PAPIIT (grants IN206009 and IN213312) and DGAPA-PASPA (to L.I.A.-A. for a research stay at IBPC). J.M.-V., D.M.D.-J., and S.Z. acknowledge receipt of CONACyT graduate fellowships.

We are pleased to acknowledge many useful discussions with Bernard Badet in the initial stages of this project. We are grateful to Mathias Springer for anti-ThrS antibodies. We thank Josef Deutscher for helpful comments on the manuscript.

FUNDING INFORMATION

This work, including the efforts of Mario Calcagno, was funded by DGAPA-PAPIIT-UNAM (IN206009 and IN213312). This work, including the efforts of Laura I. Álvarez-Añorve, was funded by DGAPA-PASPA-UNAM. This work, including the efforts of Jacqueline A. Plumbridge, was funded by Agence Nationale de la Recherche (ANR) (ANR-09-BLAN 0399 and ANR-11-LABX-0011-01). This work, including the efforts of Mario Calcagno, was funded by Consejo Nacional de Ciencia y Tecnologia (CONACYT) (99857-Q and 116074). This work, including the efforts of Jacqueline A. Plumbridge, was funded by Centre National de la Recherche Scientifique (CNRS).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

1. **Reaves ML, Young BD, Hosios AM, Xu YF, Rabinowitz JD.** 2013. Pyrimidine homeostasis is accomplished by directed overflow metabolism. Nature **500:**237–241. http://dx.doi.org/10.1038/nature12445.
2. **Calcagno M, Campos PJ, Mulliert G, Suastegui J.** 1984. Purification, molecular and kinetic properties of glucosamine-6-phosphate isomerase (deaminase) from *Escherichia coli*. Biochim Biophys Acta **787:**165–173. http://dx.doi.org/10.1016/0167-4838(84)90076-1.
3. **Midelfort CF, Rose IA.** 1977. Studies on the mechanism of *Escherichia coli* glucosamine-6-phosphate isomerase. Biochem **16:**1590–1596. http://dx.doi.org/10.1021/bi00627a010.
4. **Kochanowski K, Volkmer B, Gerosa L, Haverkorn van Rijsewijk BR, Schmidt A, Heinemann M.** 2013. Functioning of a metabolic flux sensor in *Escherichia coli*. Proc Natl Acad Sci U S A **110:**1130–1135. http://dx.doi.org/10.1073/pnas.1202582110.

5. **Mangan S, Alon U.** 2003. Structure and function of the feed-forward loop network motif. Proc Natl Acad Sci U S A **100:**11980–11985. http://dx.doi.org/10.1073/pnas.2133841100.
6. **Link H, Kochanowski K, Sauer U.** 2013. Systematic identification of allosteric protein-metabolite interactions that control enzyme activity in vivo. Nat Biotechnol **31:**357–361. http://dx.doi.org/10.1038/nbt.2489.
7. **Xu YF, Amador-Noguez D, Reaves ML, Feng XJ, Rabinowitz JD.** 2012. Ultrasensitive regulation of anapleurosis via allosteric activation of PEP carboxylase. Nat Chem Biol **8:**562–568. http://dx.doi.org/10.1038/nchembio.941.
8. **Vincent F, Davies GJ, Brannigan JA.** 2005. Structure and kinetics of a monomeric glucosamine 6-phosphate deaminase: missing link of the NagB superfamily? J Biol Chem **280:**19649–19655. http://dx.doi.org/10.1074/jbc.M502131200.
9. **Natarajan K, Datta A.** 1993. Molecular cloning and analysis of the *NAG1* cDNA coding for glucosamine-6-phosphate deaminase from *Candida albicans*. J Biol Chem **268:**9206–9214.
10. **Steimle PA, Lindmark DG, Jarroll EL.** 1997. Purification and characterization of encystment-induced glucosamine 6-phosphate isomerase in *Giardia*. Mol Biochem Parasitol **84:**149–153. http://dx.doi.org/10.1016/S0166-6851(96)02790-9.
11. **Lara-Lemus R, Calcagno ML.** 1998. Glucosamine-6-phosphate deaminase from beef kidney is an allosteric system of the V-type. Biochim Biophys Acta **1388:**1–9. http://dx.doi.org/10.1016/S0167-4838(98)00141-1.
12. **Álvarez-Añorve LI, Alonzo DA, Mora-Luga R, Lara-Gonzalez S, Bustos-Jaimes I, Plumbridge J, Calcagno ML.** 2011. Allosteric kinetics of the isoform 1 of human glucosamine-6-phosphate deaminase. Biochim Biophys Acta **1814:**1846–1853. http://dx.doi.org/10.1016/j.bbapap.2011.07.010.
13. **Horjales E, Altamirano MM, Calcagno ML, Garratt R, Oliva G.** 1999. The allosteric transition of glucosamine-6-phosphate deaminase: the structure of the T state at 2.3 Å resolution. Structure **7:**527–537. http://dx.doi.org/10.1016/S0969-2126(99)80069-0.
14. **Oliva G, Fontes M, Garratt R, Altamirano MM, Calcagno ML, Horjales E.** 1995. Structure and catalytic mechanism of glucosamine-6-phosphate deaminase from *Escherichia coli* at 2.1 Å resolution. Structure **3:**1323–1332. http://dx.doi.org/10.1016/S0969-2126(01)00270-2.
15. **Arreola R, Valderrama B, Morante ML, Horjales E.** 2003. Two mammalian glucosamine-6-phosphate deaminases: a structural and genetic study. FEBS Lett **551:**63–70. http://dx.doi.org/10.1016/S0014-5793(03)00896-2.
16. **Liu C, Li D, Liang YH, Li LF, Su XD.** 2008. Ring-opening mechanism revealed by crystal structures of NagB and its ES intermediate complex. J Mol Biol **379:**73–81. http://dx.doi.org/10.1016/j.jmb.2008.03.031.
17. **Yang C, Rodionov D, Li X, Laikova O, Gelfand M, Zagnitko O, Romine M, Obraztsova A, Nealson K, Osterman A.** 2006. Comparative genomics and experimental characterization of *N*-acetylglucosamine utilization pathway of *Shewanella oneidensis*. J Biol Chem **281:**29872–29885. http://dx.doi.org/10.1074/jbc.M605052200.
18. **Tanaka T, Takahashi F, Fukui T, Fujiwara S, Atomi H, Imanaka T.** 2005. Characterization of a novel glucosamine-6-phosphate deaminase from a hyperthermophilic archaeon. J Bacteriol **187:**7038–7044. http://dx.doi.org/10.1128/JB.187.20.7038-7044.2005.
19. **Álvarez-Añorve LI, Calcagno ML, Plumbridge J.** 2005. Why does *Escherichia coli* grow more slowly on glucosamine than on *N*-acetylglucosamine? Effects of enzyme levels, and allosteric activation on GlcN6P deaminase (NagB) on growth rates. J Bacteriol **187:**2974–2982.
20. **Plumbridge J.** 1991. Repression and induction of the *nag* regulon of *Escherichia coli* K12: the roles of *nagC* and *nagA* in maintenance of the uninduced state. Mol Microbiol **5:**2053–2062. http://dx.doi.org/10.1111/j.1365-2958.1991.tb00828.x.
21. **Gaugué I, Oberto J, Putzer H, Plumbridge J.** 2013. The use of amino sugars by *Bacillus subtilis*: presence of a unique operon for the catabolism of glucosamine. PLoS One **8:**e63025. http://dx.doi.org/10.1371/journal.pone.0063025.
22. **Álvarez-Añorve LI, Bustos-Jaimes I, Calcagno ML, Plumbridge J.** 2009. Allosteric regulation of glucosamine-6-phosphate deaminase (NagB) and growth of *Escherichia coli* on glucosamine. J Bacteriol **191:**6401–6407. http://dx.doi.org/10.1128/JB.00633-09.
23. **Montero-Morán GM, Lara-González S, Álvarez-Añorve LI, Plumbridge JA, Calcagno ML.** 2001. On the multiple functional roles of the active site histidine in catalysis and allosteric regulation of *Escherichia coli* glucosamine 6-phosphate deaminase. Biochem **40:**10187–10196. http://dx.doi.org/10.1021/bi0105835.
24. **Bustos-Jaimes I, Ramírez-Costa M, Anda-Aguilar LD, Hinjosa-Ocaña P, Calcagno M.** 2005. Evidence for two different mechanisms triggering the change in quaternary structure of the allosteric enzyme, glucosamine-6-phosphate deaminase. Biochem **44:**1127–1135. http://dx.doi.org/10.1021/bi048514o.
25. **Monod J, Wyman J, Changeux J.** 1965. On the nature of the allosteric transitions: a plausible model. J Mol Biol **12:**88–118. http://dx.doi.org/10.1016/S0022-2836(65)80285-6.
26. **Lara-González S, Dixon HBF, Mendoza-Hernandez G, Altamirano MM, Calcagno ML.** 2000. On the role of the N-terminal group in the allosteric function of glucosamine-6-phosphate deaminase from *Escherichia coli*. J Mol Biol **301:**219–227. http://dx.doi.org/10.1006/jmbi.2000.3937.
27. **Bustos-Jaimes I, Calcagno M.** 2001. Allosteric transition and substrate binding are entropy driven in Glucosamine-6-phosphate deaminase from *Escherichia coli*. Arch Biochem Biophys **394:**156–160. http://dx.doi.org/10.1006/abbi.2001.2523.
28. **Cisneros D, Montero-Morán GM, Lara-González S, Calcagno ML.** 2004. Inversion of the allosteric response of *Escherichia coli* glucosamine-6-P deaminase to *N*-acetylglucosamine 6P by single amino acid replacements. Arch Biochem Biophys **421:**77–84. http://dx.doi.org/10.1016/j.abb.2003.09.041.
29. **Bustos-Jaimes I, Sosa-Peinado A, Rudiño-Piñera E, Horjales E, Calcagno ML.** 2002. On the role of the conformational flexibility of the active site lid on the allosteric kinetics of glucosamine-6-phosphate deaminase. J Mol Biol **319:**183–189. http://dx.doi.org/10.1016/S0022-2836(02)00096-7.
30. **Plumbridge J.** 1990. Induction of the *nag* regulon of *Escherichia coli* by *N*-acetylglucosamine and glucosamine: role of the cAMP-catabolite activator protein complex in expression of the regulon. J Bacteriol **172:**2728–2735.
31. **Buescher JM, Moco S, Sauer U, Zamboni N.** 2010. Ultrahigh performance liquid chromatography-tandem mass spectrometry method for fast and robust quantification of anionic and aromatic metabolites. Analytical chemistry **82:**4403–4412. http://dx.doi.org/10.1021/ac100101d.
32. **Link H, Buescher JM, Sauer U.** 2012. Targeted and quantitative metabolomics in bacteria. Methods Microbiol **39:**127–150. http://dx.doi.org/10.1016/B978-0-08-099387-4.00005-3.
33. **Datsenko KA, Wanner BL.** 2000. One step inactivation of chromosomal genes in *Escherichia coli* K12 using PCR products. Proc Nat Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
34. **Plumbridge J.** 2009. An alternative route for recycling of N-acetylglucosamine from peptidoglycan involves the *N*-acetylglucosamine phosphotransferase system in *Escherichia coli*. J Bacteriol **191:**5641–5647. http://dx.doi.org/10.1128/JB.00448-09.
35. **Volkmer B, Heinemann M.** 2011. Condition-dependent cell volume and concentration of *Escherichia coli* to facilitate data conversion for systems biology modeling. PLoS One **6:**e23126. http://dx.doi.org/10.1371/journal.pone.0023126.
36. **Cook PF, Cleland WW.** 2007. Enzyme kinetics and mechanism. Garland Science, New York, NY.
37. **Park JT.** 2001. Identification of a dedicated recycling pathway for anhydro-*N*-acetylmuramic acid and *N*-acetylglucosamine derived from *Escherichia coli* cell wall murein. J Bacteriol **183:**3842–3847. http://dx.doi.org/10.1128/JB.183.13.3842-3847.2001.
38. **White RJ.** 1968. Control of aminosugar metabolism in *Escherichia coli* and isolation of mutants unable to degrade amino sugars. Biochem J **106:**847–858. http://dx.doi.org/10.1042/bj1060847.
39. **Gopel Y, Khan MA, Gorke B.** 2014. Menage a trois: posttranscriptional control of the key enzyme for cell envelope synthesis by a base-pairing small RNA, an RNase adaptor protein, and a small RNA mimic. RNA Biol **11:**433–442. http://dx.doi.org/10.4161/rna.28301.
40. **Göpel Y, Papenfort K, Reichenbach B, Vogel J, Görke B.** 2013. Targeted decay of a regulatory small RNA by an adaptor protein for RNase E and counteraction by an anti-adaptor RNA. Genes Dev **27:**552–564. http://dx.doi.org/10.1101/gad.210112.112.
41. **Altamirano MM, Hernandez-Arana A, Tello-Solis S, Calcagno ML.** 1994. Spectrochemical evidence for the presence of a tyrosine residue in the allosteric site of glucosamine-6-phosphate deaminase from *Escherichia coli*. Eur J Biochem **220:**409–413. http://dx.doi.org/10.1111/j.1432-1033.1994.tb18638.x.
42. **Plumbridge J.** 1998. Control of the expression of the *manXYZ* operon in

Escherichia coli: Mlc is a negative regulator of the mannose PTS. Mol Microbiol **27:**369–381. http://dx.doi.org/10.1046/j.1365-2958.1998.00685.x.

43. **Kalamorz F, Reichenbach B, Marz W, Rak B, Görke B.** 2007. Feedback control of glucosamine-6-phosphate synthase GlmS expression depends on the small RNA and involves the novel protein YhbJ in *Escherichia coli*. Mol Microbiol **65:**1518–1533. http://dx.doi.org/10.1111/j.1365-2958.2007.05888.x.
44. **Lenski RE.** 1991. Quantifying fitness and gene stability in microorganisms. Biotechnology **15:**173–192.
45. **Wiser MJ, Lenski RE.** 2015. A comparison of methods to measure fitness in *Escherichia coli*. PLoS One **10:**e0126210. http://dx.doi.org/10.1371/journal.pone.0126210.
46. **Kerfeld CA, Heinhorst S, Cannon GC.** 2010. Bacterial microcompartments. Annu Rev Microbiol **64:**391–408. http://dx.doi.org/10.1146/annurev.micro.112408.134211.
47. **Bertin Y, Chaucheyras-Durand F, Robbe-Masselot C, Durand A, de la Foye A, Harel J, Cohen PS, Conway T, Forano E, Martin C.** 2013. Carbohydrate utilization by enterohaemorrhagic *Escherichia coli* O157:H7 in bovine intestinal content. Environ Microbiol **15:**610–622. http://dx.doi.org/10.1111/1462-2920.12019.
48. **Conway T, Cohen PS.** 2015. Commensal and pathogenic *Escherichia coli* metabolism in the gut. Microbiol Spectr **3:**3. http://dx.doi.org/10.1128/microbiolspec.MBP-0006-2014.
49. **Ferenci T, Zhou Z, Betteridge T, Ren Y, Liu Y, Feng L, Reeves PR, Wang L.** 2009. Genomic sequencing reveals regulatory mutations and recombinational events in the widely used MC4100 lineage of *Escherichia coli* K-12. J Bacteriol **191:**4025–4029. http://dx.doi.org/10.1128/JB.00118-09.
50. **Baba T, Ara Y, Hasegawa M, Takai Y, Okurmura Y, Baba M, Datsenko K, Tomita M, Wanner BL, Mori H.** 2006. Construction of *Escherichia coli* K-12 in frame, single-gene knockout mutations: the Keio collection. Mol Syst Biol **2:**2006.0008.
51. **Lopez PJ, Marchand I, Joyce SA, Dreyfus M.** 1999. The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing in vivo. Mol Microbiol **33:**188–199. http://dx.doi.org/10.1046/j.1365-2958.1999.01465.x.
52. **Blangy D, Buc H, Monod J.** 1968. Kinetics of the allosteric interactions of phosphofructokinase from *Escherichia coli*. J Mol Biol **31:**13–35. http://dx.doi.org/10.1016/0022-2836(68)90051-X.